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Chapter 4: Effect of Ethanol on Hydrocarbon-degrading Bacteria in the Saturated Zone: Microbial Ecology Studies

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Summary

(Effect of Ethanol on Hydrocarbon-degrading Bacteria in the Saturated Zone: Microbial Ecology Studies)

Research was conducted to better understand how ethanol could affect the composition of subsurface microbial communities, with an emphasis on indigenous bacterial populations capable of benzene, toluene, ethylbenzene, and xylene (BTEX) degradation. Prior to this study, there were no rapid methods available for assessing the populations of anaerobic aquifer bacteria that could carry out *in situ* BTEX biodegradation. In this study, a novel DNA-based method was developed for this purpose and was used to investigate how key factors could influence the populations of toluene-degrading bacteria in microcosms incubated anaerobically with BTEX under a variety of conditions. The factors investigated included: (1) the presence or absence of ethanol, (2) the presence of various electron acceptors typical of contaminated aquifers, and (3) the fuel exposure history of the aquifer sediments used to inoculate the microcosms. The molecular method involved RTQ-PCR (Real-Time, Quantitative Polymerase Chain Reaction) analysis of a recently-discovered gene, *bssA*, that is known to be associated with the first metabolic step of anaerobic toluene degradation. Thus, analyses based on the *bssA* gene provided an estimate of the populations of anaerobic, toluene-degrading bacteria. In addition, a companion method was used to estimate total eubacterial populations using 16S rDNA.

The microcosms under study were inoculated with aquifer sediments from four sites with different histories of exposure to fuel hydrocarbons: a leaking underground fuel tank (LUFT) site at Travis Air Force Base (AFB) in California, a LUFT site in Sacramento (California), an ethanol- and fuel-contaminated terminal in the Pacific Northwest ("Northwest Terminal"), and a background, uncontaminated site in Tracy (California).

- The RTQ-PCR method developed for this study was successful at determining population trends that were consistent with observed degradation activity. The most convincing results were for Travis AFB microcosms incubated under denitrifying conditions; these microcosms clearly had the most rapid toluene degradation and the highest *bssA* abundances (representative of anaerobic toluene-degrading bacterial populations) of all the sites and conditions studied.
- Ethanol had no clear effect on the abundance of *bssA* copies in any of the microcosms analyzed. However, under the conditions tested (i.e., with electron acceptors supplied in excess), a change in numbers of *bssA* copies would not be expected because ethanol often had no discernible effect on toluene degradation activity.
- Ethanol could promote relatively minor changes in microbial ecology that could result in major changes in hydrocarbon degradation activity. The one very clear case of an effect of ethanol on toluene degradation was in Northwest Terminal microcosms incubated under denitrifying conditions; ethanol promoted toluene degradation in these microcosms. In the presence of ethanol, toluene was mostly degraded within 15 days, whereas in the absence of ethanol, there was no apparent degradation in over 50 days. The most likely explanation is that ethanol degradation fortuitously increased the populations of toluene-degrading bacteria. However, numbers of *bssA* copies were not

higher after ethanol degradation, suggesting that, if such a population increase occurred, it was within the range of experimental error.

- Ethanol could decrease the relative populations of anaerobic hydrocarbon-degrading bacteria. In some cases, ethanol was associated with marked (e.g., nine-fold) increases in total eubacterial populations but no discernible changes in *bssA* abundances or in hydrocarbon degradation activity. This indicates that ethanol, which is generally more degradable than BTEX compounds under anaerobic conditions, can disproportionately support the growth of bacteria that aren't anaerobic hydrocarbon degraders.

- Of the anaerobic electron-accepting conditions tested, denitrifying conditions were clearly the most supportive of anaerobic hydrocarbon degradation. Travis AFB microcosms incubated under denitrifying conditions had the most rapid toluene degradation and the highest *bssA* abundances of all the sites and conditions studied. Over the first four days of incubation, during which time most of the toluene and ethanol (when present) had been consumed, numbers of *bssA* copies increased 100- to 1000-fold. For Travis AFB microcosms incubated under sulfate-reducing, ferric iron-reducing, and methanogenic conditions, toluene degradation was slower and no comparable increases in *bssA* copies were detected.

- Results of this study suggest that the newly-developed, DNA-based method could provide insights into the complexity of *in situ* microbial ecology of fuel-contaminated aquifers, but that further developments would be beneficial. For example, some of the results in this study are a reminder that genetic potential (represented by DNA) is not always realized and that genetic expression (represented by mRNA) is also important. To illustrate, Sacramento aquifer material (before incubation) had the highest absolute numbers of *bssA* copies of any aquifer material analyzed. However, the toluene degradation rates of Sacramento denitrifying microcosms were comparable to those of Tracy denitrifying microcosms, which had by far the lowest absolute numbers of *bssA* copies. The method developed in this study could be modified to quantify gene expression by using mRNA. Ideally, both DNA and mRNA approaches could be used to assess the populations and activities of hydrocarbon-degrading bacteria in the subsurface.

- To evaluate whether these laboratory microcosm results can be extrapolated to actual gasohol releases at LUFT sites, this molecular ecology approach should be used to characterize the spatial and temporal changes in subsurface microbial communities as a result of controlled gasohol release field experiments.

- If enhanced *in situ* bioremediation is considered at sites contaminated with gasohol, addition of nitrate as an electron acceptor could produce positive results. Denitrifying conditions were most favorable for anaerobic BTEX degradation for all sites tested, and, for Travis AFB microcosms, supported the rapid growth of toluene-degrading bacteria. These results are consistent with the findings of other BTEX biodegradation studies. Nitrate addition is easier to implement than oxygen addition because nitrate is much more soluble in water and is not a gas. Concerns regarding the use of nitrate include the following: (1) it is regulated in groundwater, (2) nitrogen gas formed by denitrification could create bubbles and disrupt groundwater flow, and (3) the degradation of benzene, the most toxic of the BTEX compounds, may not occur under denitrifying conditions at all sites. These concerns need not disqualify *in situ* nitrate addition, as added nitrate concentrations could be kept below regulatory guidelines and *in situ* benzene degradation cannot be guaranteed under any electron-accepting conditions.

4. Effect of Ethanol on Hydrocarbon-degrading Bacteria in the Saturated Zone: Microbial Ecology Studies

4.1. Introduction

Concerns that leaking underground fuel tanks (LUFTs) would promote widespread groundwater contamination by methyl tertiary butyl ether (MTBE), a fuel oxygenate, have prompted California to mandate the removal of MTBE from gasoline by December 31, 2002 (Executive Order D-5-99, Governor Gray Davis). Ethanol is being considered as a replacement oxygenate that would address air quality objectives without seriously deteriorating groundwater quality. As part of a state-funded effort to assess the potential environmental effects of using gasohol (i.e., gasoline amended with ethanol), laboratory microcosm and aquifer column studies were conducted to examine the effects of ethanol on the intrinsic biodegradation of benzene, toluene, ethylbenzene, and xylenes (BTEX), which are the most water-soluble and toxic hydrocarbon components of unleaded gasoline. Chapter 3 (Alvarez *et al*., 2001) described how ethanol affected the kinetics of BTEX degradation by aquifer bacteria from a range of sites under a range of electron-accepting conditions. In this chapter, we describe how ethanol affected the composition of microbial communities in those microcosms, with an emphasis on bacterial populations capable of anaerobic BTEX degradation. This represents an effort to better understand the ecological changes underlying the observed effects of ethanol on BTEX degradation kinetics.

The potential effects of ethanol on intrinsic BTEX degradation include the following: (1) preferential degradation of ethanol could lead to depletion of the most thermodynamically favorable electron acceptors (such as oxygen or nitrate), resulting in anaerobic conditions characterized by slow BTEX degradation rates (e.g., Powers *et al*., 2001), (2) preferential degradation of ethanol could lead to depletion of essential nutrients, thus limiting the growth of bacteria capable of BTEX degradation, (c) degradation of ethanol could fortuitously increase the populations of bacteria capable of BTEX degradation, thus enhancing degradation rates, (3) at concentrations above a toxicity threshold, ethanol could inhibit or kill bacteria capable of BTEX degradation. Aquifer column studies were used to address (a) (see Chapter 3, Alvarez *et al*., 2001). The experimental design of this microcosm study eliminated (a) and (d), respectively, by providing single electron acceptors in excess and by using a relatively low but realistic ethanol concentration. This experimental design was used to simplify data interpretation.

Since the goal of this microcosm study was to investigate the effects of ethanol on BTEX-degrading bacteria, it was necessary to characterize the bacterial communities in terms of their BTEX-degrading capabilities rather than in terms of their species composition (phylogeny), which is the conventional approach for microbial characterization. Techniques of molecular biology were used because they preclude laboratory isolation and cultivation of bacteria, which can be extremely time-consuming and often unsuccessful at including the majority of bacterial species in environmental samples. To date, molecular ecology techniques have focused overwhelmingly on phylogeny, primarily because of success in the use of 16S rRNA (or 16S

rDNA) to identify bacterial species in environmental samples (e.g., Olsen *et al.*, 1986; Amann *et al*., 1995; Muyzer *et al.*, 1993). Such molecular phylogenetic approaches could only identify changes in species composition as a result of the presence of ethanol, which would be of very limited use in explaining changes in BTEX degradation.

We developed a culture-independent, molecular method to detect and quantify bacteria based on their genetic potential to degrade certain BTEX compounds. Development of this method was predicated on the following generalizations about intrinsic biodegradation of BTEX compounds at LUFT sites: (a) anaerobic conditions typically prevail, as indigenous, hydrocarbon-degrading, aerobic bacteria readily deplete dissolved oxygen (e.g., Lee *et al.*, 1988)—oxygen would be even more readily depleted in the presence of ethanol, which is highly biodegradable, and (b) toluene is the most readily degradable of the BTEX compounds under anaerobic conditions (e.g., Heider *et al*., 1998; Spormann and Widdel, 2000; Alvarez *et al*., 2001). Thus, the method focused on detecting bacteria with the genetic potential to carry out anaerobic toluene degradation.

Understanding of the biochemistry and genetics of anaerobic toluene degradation has increased dramatically since 1996. A novel enzyme that catalyzes the first step of anaerobic toluene degradation, benzylsuccinate synthase (BSS), has been reported in cultures that degrade toluene under denitrifying (Beller and Spormann, 1997a; Biegert *et al*., 1996), sulfate-reducing (Beller and Spormann, 1997b; Rabus and Heider, 1998), anoxygenic phototrophic (Zengler *et al*., 1999), and methanogenic (Beller and Edwards, 2000) conditions; all these studies involved *in vitro* enzyme assays. The reaction mechanism of this enzyme is very different than that of the oxygenases that catalyze *aerobic* toluene degradation; in fact, molecular oxygen, which is a critical substrate of oxygenases, strongly deactivates BSS (Heider *et al*., 1998). BSS has been purified from two denitrifying bacteria (Leuthner *et al.*, 1998; Beller and Spormann, 1999) and the gene encoding for the large (α) subunit of BSS (*bssA*) has been sequenced from two strains of the denitrifying bacterium *Thauera aromatica* (Leuthner *et al.*, 1998, Coschigano *et al*., 1998). Notably, BSS (or a closely related enzyme) has recently been shown to catalyze the first step of anaerobic xylene degradation (Krieger *et al*., 1999). Thus, detection of bacteria harboring genes for BSS may be relevant to anaerobic xylene as well as toluene degradation. To date, no enzyme other than BSS has been identified that catalyzes the first (activation) step of anaerobic toluene degradation. Although another activation step was proposed for *Azoarcus tolulyticus* strain Tol-4 (Chee-Sanford *et al.*, 1996), the proposed enzyme activity has never been demonstrated during *in vitro* experiments, and in fact, this strain has been shown to contain BSS activity (Beller and Spormann 1997b).

Real-Time, Quantitative Polymerase Chain Reaction (RTQ-PCR) analysis was chosen to quantify *bssA* and 16S rRNA genes because this recently developed technology offers advantages over more conventional methods used to detect specific bacterial populations in environmental samples, such as DNA:DNA hybridization (Stapleton *et al*., 2000). A primary disadvantage of membrane-based, DNA:DNA hybridization methods, such as slot blot hybridization, is lack of sensitivity: detection limits are on the order of 10^5 or greater for functional genes, which typically occur at a frequency of only one copy per cell. Quantification of low abundance gene targets in complex DNA extracts is even more problematic, given the limited capacity of membranes used for hybridization. The PCR (Saiki *et al*., 1988) has been used to increase the quantity of low abundance genes for detection. Most PCR-based quantification methods, such as denaturing gradient gel electrophoresis (DGGE, Muyzer *et al*.,

1993), and terminal restriction fragment length polymorphism analysis (TRFLP, Liu *et al*., 1997), rely on amplification of target DNA to a sufficient concentration to allow end-point detection. However, studies have shown that accumulation of amplification products can bias the proportions of amplified DNA in mixtures relative to the proportions in the original sample (Suzuki and Giovannoni, 1996). The RTQ-PCR method precludes problems associated with end-point detection by quantifying amplified DNA in an early, exponential phase of the reaction (Livak *et al*., 1995). The method is especially suited to quantification of low abundance gene targets, even in complex DNA extracts. Other advantages of RTQ-PCR are that quantitative data are rapidly collected in real-time during the amplification process and the linear range often encompasses over five orders of magnitude. Although most RTQ-PCR applications to date have involved the detection and quantification of pathogenic bacteria in food or animal tissue, the technique has recently been used to quantify specific phylogenetic groups of bacteria in environmental samples based on their 16S rDNA (Suzuki *et al*., 2000).

In this study, we used RTQ-PCR to investigate how certain factors could influence the populations of toluene-degrading bacteria in microcosms incubated anaerobically with BTEX under a variety of conditions. The factors investigated include: (1) the presence or absence of ethanol, (2) the presence of various electron acceptors, and (3) the fuel exposure history of the aquifer sediments used to inoculate the microcosms. RTQ-PCR analysis was used to quantify the populations of bacteria containing the *bssA* gene as well as populations of total eubacteria (as represented by a universal eubacterial probe based on 16S rDNA). We address the significance of this molecular ecology approach to understanding the potential effects of gasohol spills on intrinsic BTEX degradation.

4.2. Materials and Methods

4.2.1. Microcosm Construction and Sampling

Microcosms were constructed as described in Chapter 3 (Alvarez *et al*., 2001). Briefly, the microcosms (constructed in duplicate) consisted of 40 mL of artificial groundwater and 10 g of homogenized aquifer sediment; they were contained in 125-mL amber glass bottles sealed with Teflon Mininert valves (Alltech Associates, Inc.) and were prepared and incubated under anaerobic conditions in an anaerobic glove box. Microcosms were amended with BTEX (0.4 to 3 mg/L for individual BTEX compounds), ethanol (50 to 100 mg/L, when added), and various electron acceptors (either nitrate, sulfate, or amorphous ferric oxyhydroxide; bicarbonate was present as a buffer in all microcosms and was intended to support methanogenic conditions when no other electron acceptor was added). Details about the conditions tested at each site are presented in Chapter 3 (Alvarez *et al*., 2001).

Molecular analyses focused only on microcosms for which there was strong evidence of biological toluene removal. Thus, eight sets of microcosms were analyzed (a "set" is defined here as a group of microcosms inoculated with aquifer sediment from a given site under given electron-accepting conditions, in both the presence and absence of ethanol). In general, microcosm sampling was designed to investigate the effects of ethanol on the populations of bacteria carrying the *bssA* gene and of total eubacteria. There were four sampling times for each set of microcosms:

- Control—Microcosms sacrificed without incubation (initial conditions). There was only one group of anaerobic controls for each site, which applied to all electron-accepting conditions used for that site.
- I—When ethanol was depleted in the ethanol-amended microcosms, both the ethanol-amended and unamended microcosms were sampled. These samples provided direct evidence of the effect of ethanol degradation on bacterial populations incubated under given conditions.
- II—Microcosms were sampled when the first BTEX compound (almost always toluene) was depleted (either in the ethanol-amended or unamended microcosms, whichever degraded BTEX more quickly).
- III—Microcosms were sampled when the first BTEX compound (almost always toluene) was depleted (either in the ethanol-amended or unamended microcosms, whichever degraded BTEX more slowly).

Samples II and III were intended as a means of "calibrating" the *bssA* results because they indicated the amount of increase in numbers of *bssA* copies after a known amount of toluene had been completely depleted. Thus, if ethanol alone was observed to increase the number of *bssA* copies at time I, this increase could be compared to the increases observed at times II and/or III.

Sampling times for specific microcosms were determined by monitoring a parallel "analytical" set of microcosms incubated under the same conditions (see Chapter 3, Alvarez *et al*., 2001). When ethanol or toluene was observed to be depleted in the "analytical" microcosms, the corresponding microcosms were tested a day later to confirm that the compound was depleted, and if confirmed, the microcosms were sacrificed for molecular analysis. Centrifugation was used to harvest microcosms for molecular analysis, as described in Chapter 3 (Alvarez *et al*., 2001). Exact sampling times are given in Chapter 3, Appendix E (Alvarez *et al*., 2001). Microcosm samples were stored at -80°C prior to DNA extraction.

There were two cases in which sampling deviated from the above procedure. For Travis AFB microcosms incubated without ethanol under denitrifying conditions, sample I was collected on Day 12 instead of Day 4. In addition, for Travis AFB microcosms incubated without ethanol under sulfate-reducing conditions, sample I was collected on Day 33 instead of Day 15.

4.2.2. DNA Extraction

The DNA extraction protocol was modified from Zhou *et al.*, (1996). Five g of wet sediment from each microcosm was extracted three times using sodium dodecyl sulfate and heating at 65°C. Prior to extraction, PCR Inhibitor Removal Solution (IRS; Mo Bio, Carlsbad, CA) was added at a 10% final concentration. In addition, 10 ng of a 500-bp PCR product (bp 7131-7630) amplified from Bacteriophage Lambda was added as an internal standard to assess DNA recovery on a sample-specific basis (Johnston *et al*., 1996). The supernatants from each of the three extractions were pooled and subjected to chloroform extraction. Phase-lock gel (Heavy, Eppendorf AG, Hamburg, Germany) was added prior to centrifugation in order to form a solid interface between the upper aqueous phase (which contained the DNA) and the lower organic phase; thus, the upper phase was recovered by simply decanting. DNA was precipitated from samples by adding isopropanol (0.6 vol.) and linear acrylamide (25 µg, Ambion, Austin, TX) to

facilitate precipitation, incubating for one hr and centrifuging at 14,000 rpm for 20 min. at room temperature. The DNA pellet was dried under vacuum (Speed Vac DNA120, Savant, Farmingdale, NY) and resuspended in 500 µL TE buffer (Tris-OH, 10 mM; EDTA, 1 mM; pH 8). The crude DNA extract was further purified and concentrated five-fold using a Mo Bio Spin Filter following the manufacturer's protocols.

4.2.3. Real-Time, Quantitative Polymerase Chain Reaction (RTQ-PCR)

The RTQ-PCR method uses the 5'-nuclease activity of *Taq* DNA polymerase (from *Thermus aquaticus*) to cleave an internal dual-labeled "probe" annealed to the target DNA sequence as it polymerizes through the region. The probe has two fluorescent dyes, a "reporter" at the 5' end and a "quencher" at the 3' end. Energy absorbed by the reporter is transferred to the quencher when the two dyes are close in proximity. However, when the probe is cleaved, the quencher is no longer associated with the reporter and fluorescence is emitted. The amount of fluorescence is proportional to the amount of PCR product made. The instrument identifies a cycle threshold, C_t, which is the PCR cycle at which the fluorescence emitted exceeds a threshold value set at 10 times the standard deviation of the background fluorescence of the sample. The amount of gene target originally present in the sample is then determined by reference to a calibration curve of log target concentration versus C_t.

Primers and probes were designed based on a thorough investigation of sequences of *bssA* in a variety of toluene-degrading bacteria, including four denitrifiers [*Azoarcus* sp. strain T (Krieger *et al*., 1999), *Thauera aromatica* strains T1 and K172 (Coschigano *et al*., 1998; Leuthner *et al.,* 1998), and *Azoarcus tolulyticus* strainTol-4 (Chee-Sanford *et al*., 1996)] and sulfate-reducing strain PRTOL1 (Beller *et al*., 1996). The *bssA* sequences for strains T1 and K172 are available in the GenBank database, whereas those for strains T, Tol-4, and PRTOL1 were determined in this study by designing PCR primers based on strains T1 and K172. After PCR amplification, the putative *bssA* sequences were cloned and sequenced and aligned with known *bssA* sequences to determine consensus regions for RTQ-PCR primer and probe design. Two sets of *bssA* primers and probes were designed: one based on the four denitrifying bacteria (the "*denitrifier*" primer/probe set) and one based on sulfate-reducing strain PRTOL1. The "denitrifier" 28 bp fluorogenic probe was 100% homologous to *bssA* genes in all four denitrifiers, whereas the 18 bp PCR primers were degenerate, containing one or two alternate bases in order to be homologous to all four *bssA* genes in the denitrifying bacteria. The 130 bp amplicon (i.e., the total amplified region defined by the primers) was 85% identical between the four denitrifiers. Primer and probe selection was empirically based on an observed region of high homology rather than on either of two regions putatively associated with the catalytic activity of BssA (Coschigano *et al.,* 1998; Leuthner *et al.,* 1998). Primers and probes were tested against genomic DNA from strains K172, T, and Tol-4, and *E. coli* as a negative control. *E. coli* DNA was used as a negative control because it contains a gene coding for pyruvate formate-lyase (PFL), which has strong homology to the C-terminal region of BssA (the protein encoded for by *bssA*). Despite its high homology to BSS, PFL plays no role in anaerobic toluene metabolism (it catalyzes the homolytic cleavage of pyruvate to form acetyl coenzyme A and formate). The second primer/probe set for *bssA* was based on a region similar to that of the "denitrifier" set, but it was based solely on the *bssA* sequence of sulfate-reducing strain PRTOL1 (with no degeneracy), and was intended for quantification of toluene-degrading, sulfate-reducing bacteria. The two fluorogenic probes (i.e., the "denitrifier" probe and strain PRTOL1 probe) had 3 base differences between them. Based

on results with negative controls involving *E. coli*, only the "denitrifier" primer/probe set was used in this study.

Reactions were also performed using primers and a probe for 16S rDNA of eubacteria, after Suzuki *et al.* (2000). Specifically, the primers used were BACT1369F and PROK1492R, and the probe was TM1389F. This primer/probe set was shown to be highly inclusive of bacterial 16S rDNA in environmental samples (Suzuki *et al.,* 2000). RTQ-PCR primers and a probe specific to Bacteriophage Lambda were used to quantify the recovery of the internal standard in a separate RTQ-PCR analysis.

A 5 µL volume of extracted, undiluted DNA (*bssA*) or 10-fold diluted DNA (16S rDNA or Lambda) was subjected to RTQ-PCR using a Smart Cyclerâ System (Cepheid, Sunnyvale, CA). The PCR mixture contained 0.625 µM of each primer, either 0.2 µM probe (*bssA* or 16S rDNA) or 0.195 µM probe (Lambda), 1.25 U Taq Polymerase and 1X PCR Buffer A (Fisher Scientific, Pittsburgh, PA), 1 mM of each deoxynucleoside triphosphate, 1X Cepheid additive reagent [0.2 mg/mL Bovine Serum Albumin (BSA), 150 mM trehalose, 0.2% Tween 20, and 0.2 mM Tris Buffer, pH 8.0] and sterile water to give a final volume of 25 µL. For amplification of 16S rDNA, BSA and trehalose were omitted because they contributed to the fluorescence background in negative control reactions that lacked an added template. The additive helped to prevent reactants and products from adhering to the sides of the PCR tubes, which have a large surface area. In our practice, the additive was necessary to obtain consistent results with low detection limits down to 100 gene targets, but did not significantly influence results for $\geq 10^4$ targets. The PCR conditions were as follows: 95°C for 10 min; 50 cycles of 95°C for 15 s and 58°C for 60 s. The melting temperatures of primers were about 65°C whereas that of the probe was about 72°C. For all sample batches, a negative control reaction without template was run to verify that contaminating DNA was not introduced.

4.2.4. Data Analysis

Dilutions of strain K172 genomic DNA (10^2 to 10^7 copies of *bssA*) were used to generate calibration curves for *bssA* quantification. The log concentration of *bssA* copies was plotted versus the cycle threshold (C_t). The following assumptions were used in calculating the number of *bssA* copies in a sample: (1) the approximate size of the strain K172 genome was 3×10^6 bp; (2) there was one copy of *bssA* per genome; (3) the *bssA* calibration curve for strain K172 was representative of other bacteria containing *bssA*. Similar calibration curves were used for 16S rDNA; in this case, genomic DNA from *E. coli* strain DH5α was used for standard dilutions (10^4 to 10^8 copies of 16S rDNA), and analogous assumptions were used for quantification. The last assumption is necessary given the genetic diversity that will inevitably be encountered in environmental samples (e.g., the natural diversity in *bssA* sequences in all toluene-degrading, anaerobic bacteria). To the extent that these assumptions are inaccurate, *bssA* and 16S rDNA data must be considered as estimates. Nonetheless, the data are suitable for determining relative differences in gene abundances as a function of variation in the conditions of microcosm incubation. The RTQ-PCR analysis of the internal standard Lambda was used to correct the *bssA* and 16S rDNA data for efficiency of DNA recovery during the extraction process. Specifically, when the recovery of Lambda was <100%, *bssA* and 16S rDNA data were normalized to correct for recovery. However, no correction was made for samples in which Lambda recoveries exceeded 100%. Unlike the quantification method used for *bssA* and 16S

rDNA, no assumptions had to be made for Lambda quantification because the Lambda stock solution contained DNA fragments of known length (not genomic DNA) and natural genetic diversity was not an issue (Lambda DNA added to samples was identical to that in the standard stock solution).

4.2.5. Analyses of Toluene and Ethanol

Toluene and ethanol were analyzed as described in Chapter 3 (Alvarez *et al*., 2001).

4.3. Results

4.3.1. Performance of RTQ-PCR Methods Developed for this Study

4.3.1.1. *bssA Primers/Probes*

Representative PCR amplification progress curves for *bssA* standards are presented in Figure 4-1(A). These data include standards ranging from 10^2 to 10^5 target copies along with a no-target control. Figure 4-1(B) shows the inverse relationship between log target concentration and C_t for the standards. For each sample batch analyzed by RTQ-PCR, standard curves used for quantification included four to five data points and encompassed the C_t range of microcosm samples. Calibration curves for *bssA* were highly linear ($r^2 \geq 0.992$) over the range of 10^2 to 10^7 copies per reaction. The operational detection limit for *bssA* was determined to be ca. 50 targets per sample; therefore, the method detection limit was 2×10^3 targets per sample (accounting for 40-fold dilution of samples for *bssA*). The no-target control, which was analyzed along with each batch of samples, always gave a similar result to that shown in Figure 4-1(A), demonstrating that target DNA contamination was not introduced into RTQ-PCR reactions.

The universality of the "denitrifier "primer/probe set for detecting *bssA* in denitrifying bacteria was determined by testing dilutions of genomic DNA from three toluene-degrading, denitrifying bacterial strains (*T. aromatica* strain K172, *A. sp.* strain T, and *A. tolulyticus* strainTol-4). The *bssA* calibration slopes for the three strains were similar (4.3% relative standard deviation) over the range of 10^3 to 10^6 genome copies, which indicates that the calibration based on strain K172 used in this study would accurately quantify *bssA* in at least two other toluene-degrading, denitrifying strains (within one order of magnitude). However, the "denitrifier" primer/probe set was unable to detect ca. 10^5 copies of *bssA* in genomic DNA from toluene-degrading, sulfate-reducing strain PRTOL1, suggesting that this primer/probe set could be inefficient at detecting *bssA* in sulfate-reducing bacteria and potentially in other physiological groups as well. In relation to the PRTOL1 *bssA* sequence (determined in this study), the "denitrifier" primers each have two base pair mismatches and the probe has three base pair mismatches. Finally, the specificity of the "denitrifier" primer/probe set for *bssA* quantification was tested by using 10^5 genome copies of *E. coli* as a negative control. As was the case for no-template controls, no increase in fluorescence was detected, demonstrating that there was no cross-reactivity with the *pfl* gene (or any other gene) that is present in *E. coli*.

In light of the poor performance of the "denitrifier" primer/probe set for sulfate-reducing strain PRTOL1, attempts were made to develop a primer/probe set for quantitative analysis of toluene-degrading, sulfate-reducing bacteria based on this strain. However, in contrast to the

"denitrifier" primer/probe set, the strain PRTOL1 primer/probe set showed strong cross-reactivity with *E. coli* DNA and yielded a similar C_t value for *E. coli* and strain PRTOL1. A suitable primer/probe set for *bssA* in sulfate-reducing bacteria could not be developed in time for use in this project.

4.3.1.2. 16S rDNA and Lambda Primers/Probes

Similar calibration curves were generated for 16S rDNA and Lambda as those described for *bssA* quantification, with standards ranging from 10^4–10^8 genome copies of *E. coli* or copies of the 500-bp Lambda fragment, respectively. Calibration curves for 16S rDNA and Lambda were linear ($r^2 \geq 0.992$). The no-template controls for 16S rDNA analysis were always non-detect (no fluorescence increase), whereas those for Lambda analysis occasionally had cycle thresholds $\geq$40. The response for Lambda no-template controls was negligible, as Lambda copies present in microcosm samples were >7 orders of magnitude higher than those calculated for the no-template control.

4.4. Travis AFB Microcosms

Microcosms inoculated with aquifer sediment from Travis AFB had greater toluene degradation rates than microcosms from the other three sites tested, particularly under denitrifying conditions (see Chapter 3, Alvarez *et al*., 2001).

4.4.1. Denitrifying Conditions

As shown in Figure 4-2(A), ethanol did not appear to affect toluene degradation rates under denitrifying conditions. Since ethanol and toluene were degraded concurrently, and the toluene degradation rate was similar in the presence and absence of ethanol, it was not possible to collect samples that could definitively show the effect of ethanol alone on *bssA* abundances. A very steep increase in the number of *bssA* copies occurred between the initial conditions (i.e., the controls) and the first sampling when ethanol (and most of the toluene) had been degraded: copies of *bssA* increased from ca. 10^6 initially to between 10^8 and 10^9 at sampling point I (Figure 2[B]). The numbers of *bssA* copies did not vary markedly among samples I–II (2.2% relative standard deviation based on log units), and thus, the presence of ethanol had no clear effect on *bssA* abundance.

In the absence of ethanol, the numbers of copies of 16S rDNA (intended to represent trends in the total eubacterial population) did not change notably from the initial value of ca. 2×10^9 (Figure 4-2[B]). However, for ethanol-amended microcosms, there was an increase of a factor of 4 to 5 relative to the initial conditions.

The relative abundance of *bssA* copies (i.e., copies of *bssA* relative to copies of 16S rDNA) was surprisingly high, ca. 65%, for the microcosms that contained no ethanol, whereas this ratio was much lower, ca. 5%, in samples that were amended with ethanol, and in the controls (<0.1%). The simplest interpretation is that ethanol, which can be degraded by a wider variety of bacteria than toluene under anaerobic conditions, served as a growth substrate for many bacteria that did not carry the *bssA* gene.

Recoveries of the surrogate (Lambda) ranged from 49–101% for all replicates represented in Figure 4-2(B).

4.4.2. Sulfate-reducing Conditions

Toluene degradation under sulfate-reducing conditions was somewhat slower than under denitrifying conditions, and it is possible that ethanol slightly enhanced the toluene degradation rate (Figure 4-3[A]). Concurrent degradation of toluene and ethanol was observed under sulfate-reducing conditions (Figure 4-3[A]) as it was for denitrifying conditions. At sampling time I, when ethanol and a large portion of toluene were degraded, abundances of *bssA* averaged about 175% those of the initial conditions (controls), regardless of whether or not ethanol was present (Figure 4-3[B]). By sampling point III, by which time toluene and *m,p*-xylenes (Chapter 3, Alvarez *et al.*, 2001) had been degraded in the absence of ethanol, the abundance of *bssA* had increased to about 250% of the abundance of the controls. Notably, benzylsuccinate synthase (and therefore, *bssA*) may be involved in anaerobic xylene degradation as well as toluene degradation (Krieger *et al.*, 1999), so the degradation of *m,p*-xylenes could have affected the abundance of *bssA.*

Ethanol may have enhanced the abundance of total bacteria (as represented by 16S rDNA), but this effect is less certain than it was under denitrifying conditions. Although the abundance of 16S rDNA was, on average, 5 to 6 times greater in the presence of ethanol than in its absence in samples I to III, the ethanol-amended microcosms had approximately the same abundance of 16S rDNA as the control (Figure 4-3[B]).

The abundance of *bssA* copies relative to 16S rDNA copies was much lower under sulfate-reducing conditions than under denitrifying conditions (ca. 0.1 to 2% in samples I to III). Based on the relatively poor efficiency with which the *bssA* primers and probe used in this study detected *bssA* in a sulfate-reducing, toluene-degrading bacterium (10^5 copies of *bssA* were not detected; see Materials and Methods), it is plausible that the actual abundances of *bssA* were underestimated under sulfate-reducing conditions.

Recoveries of the surrogate (Lambda) ranged from 58–120% for all replicates represented in Figure 4-3(B) (recall that recovery corrections for *bssA* and 16S rDNA were not made for samples with Lambda recoveries >100%).

4.4.3. Methanogenic Conditions

Toluene degradation under methanogenic conditions appeared to be biphasic, with a more rapid initial phase during which ca. 50–75% of the toluene was degraded, followed by a much slower phase (Figure 4-4[A]). It is possible that the initial phase represented sulfate-reducing conditions (as a low concentration of sulfate was present in the artificial groundwater), and the slower phase represented true methanogenic conditions after the sulfate had been depleted.

There were no discernible changes in *bssA* abundances relative to controls in samples I to III (Figure 4-4[B]). Abundances of 16S rDNA in samples I to III averaged 200% those of the controls; in contrast to trends under denitrifying and sulfate-reducing conditions, there was no evidence that ethanol affected the numbers of 16S rDNA copies. Notably, the 16S rDNA primers and probes used in this study targeted eubacteria, not archaea, so methanogens would not be included in the 16S rDNA counts. The abundance of *bssA* copies relative to 16S rDNA copies was much lower than under sulfate-reducing or denitrifying conditions (<0.1% in samples I to III).

Recoveries of the surrogate (Lambda) ranged from 82–169% for all replicates represented in Figure 4-4(B).

4.4.4. Ferric Iron-amended Conditions

Trends of toluene and ethanol degradation were similar under iron-amended and methanogenic conditions, although toluene removal in the absence of ethanol was more efficient under iron-amended conditions (Figure 4-5[A]). There were no marked changes in the abundances of *bssA* or 16S rDNA in samples I to III as compared to the control, and thus no detectable effects of ethanol. The ratios of *bssA* copies relative to 16S rDNA copies were comparable to the values under methanogenic conditions (<0.1% in samples I to III).

Recoveries of the surrogate (Lambda) ranged from 42–128% for all replicates represented in Figure 4-5(B).

4.5. Northwest Terminal Microcosms

Anaerobic toluene degradation was observed under denitrifying and sulfate-reducing conditions in microcosms inoculated with aquifer sediment from the Northwest Terminal (Chapter 3, Alvarez *et al*., 2001). Toluene degradation rates were generally slower than those observed for microcosms from Travis AFB.

4.5.1. Denitrifying Conditions

Ethanol had a dramatic and positive effect on anaerobic toluene degradation under denitrifying conditions (Figure 4-6[A]). In the presence of ethanol, toluene was mostly degraded by Day 15, whereas in the absence of ethanol, there was no clear degradation in over 50 days (Figure 4-6[A]). All of the three replicates for ethanol-amended and unamended conditions followed the trends shown. Notably, nitrate was depleted in the ethanol-amended microcosms between Days 5 and 8 and was re-amended on Day 10 (Chapter 3, Alvarez *et al*., 2001), so it is likely that toluene degradation would have begun several days sooner if nitrate had not been depleted.

If ethanol promoted toluene degradation by supporting the growth of anaerobic, toluene-degrading bacteria, this is not reflected in the data for *bssA*. Indeed, the numbers of *bssA* copies at sampling time I (when ethanol was degraded) were three to four times higher in the absence of ethanol than in its presence (Figure 4-6[B]). Unfortunately, control (i.e., initial) data for *bssA* and 16S rDNA could not be generated because some compound(s) in the extracts of these autoclaved samples strongly inhibited the DNA polymerase used for PCR amplification (e.g., Lambda recoveries in the controls ranged from only 0.1 to 11%). Comparison of the 16S rDNA data for samples I and II show no clear effect of ethanol (Figure 4-6[B]). The ratios of *bssA* copies relative to 16S rDNA copies were very low (typically ≤0.001%), even in the samples with toluene degradation, as compared to values observed in Travis AFB microcosms (Figure 4-6[B]).

Recoveries of the surrogate (Lambda) ranged from 59–127% for all replicates represented in Figure 4-6(B).

4.5.2. Sulfate-reducing Conditions

Toluene degradation was observed under sulfate-reducing conditions (Figure 4-7[A]). Although it appears that ethanol lengthened the lag period before degradation commenced, this may be an indirect effect related to the consumption of sulfate during ethanol degradation; sulfate was depleted in ethanol-amended microcosms between Days 10 and 17, at which time it was re-amended (Chapter 3, Alvarez *et al*., 2001). Thus, the ethanol-amended microcosms were without sulfate for one week, which is approximately the additional length of the lag period for the ethanol-amended microcosms relative to the unamended microcosms (Figure 4-7[A]).

There were no marked differences in the numbers of *bssA* copies as a function of the presence of ethanol (Figure 4-7[B]), although the *bssA* values in the ethanol-amended microcosms were about twice those of the microcosms without ethanol at sampling time I (when ethanol was depleted). As discussed previously, control data are not available for the Northwest Terminal microcosms. There were also no clear effects of ethanol on the abundance of 16S rDNA copies (Figure 4-7[B]). The ratios of *bssA* copies relative to 16S rDNA copies were $\leq$0.01%.

Recoveries of the surrogate (Lambda) were high, 123–245%, for all replicates represented in Figure 4-7(B); thus, none of the *bssA* or 16S rDNA data were recovery-corrected.

4.6. Sacramento Microcosms

4.6.1. Denitrifying Conditions

Anaerobic toluene degradation was observed under denitrifying conditions for microcosms inoculated with aquifer sediment from a LUFT site in Sacramento, however, the degradation rate was considerably slower than that observed for Travis AFB or Northwest Terminal microcosms (Figure 4-8[A]). In ethanol-amended microcosms, the toluene degradation rate appeared to increase after approximately one month (Figure 4-8[A]).

Ethanol degradation may have slightly increased the number of *bssA* copies; at sampling time I (after ethanol was degraded), the numbers of *bssA* copies in ethanol-amended microcosms were approximately 170% those in unamended microcosms (Figure 4-8[B]). However, there was not a wide range of *bssA* values overall, including the controls (Figure 4-8[B]). Numbers of 16S rDNA copies increased for samples I to II relative to controls, and the increase was greatest for ethanol-amended microcosms (Figure 4-8[B]). Abundances of 16S rDNA copies in ethanol-amended microcosms were, on average, approximately 50 times those of the controls and 9 times those of the unamended microcosms. The ratios of *bssA* copies relative to 16S rDNA copies varied from approximately 3% in the controls, to 0.5% in the unamended microcosms, to $\leq$0.1% in the ethanol-amended microcosms.

Recoveries of the surrogate (Lambda) were uniformly low, 25–41%, for all replicates represented in Figure 4-8(B).

4.7. Tracy Microcosms

The Tracy site was chosen to be representative of aquifer materials that have not been exposed to leaking underground fuel tanks.

4.7.1. Denitrifying Conditions

Relatively slow toluene degradation was observed under denitrifying conditions, and ethanol had no apparent effect on the rate (Figure 4-9[A]).

Numbers of *bssA* copies in samples I to III were roughly equal to or lower than numbers in the control (initial) samples (Figure 4-9[B]). The variability apparent in *bssA* values among samples may be due to the fact that they are approaching detection limits in this sample matrix (e.g., *bssA* was not detectable in one replicate of sample III, despite the favorable 85% Lambda recovery in this sample). Numbers of 16S rDNA copies may also have been relatively close to detection limits, as this gene was barely detectable in both of the control replicates. It appears that ethanol enhanced the numbers of 16S rDNA copies, as the average abundance in ethanol-amended microcosms was ca. 5.5 times higher than the average in the unamended microcosms. The ratios of *bssA* copies relative to 16S rDNA copies were <0.02% in all samples in which both variables were detected. The detection of *bssA* in aquifer material that has not been exposed to leaking underground fuel tanks is not particularly surprising, as toluene-degrading, denitrifying bacteria have been found in a range of pristine environments (Fries *et al.,* 1994).

Recoveries of the surrogate (Lambda) ranged from 21–85% for all replicates represented in Figure 4-9(B).

4.8. Discussion

The molecular method developed for this study provided some insight into the relationships between microbial ecology and anaerobic hydrocarbon degradation under a range of conditions. The most dramatic results were for Travis AFB microcosms incubated under denitrifying conditions; these microcosms clearly had the most rapid toluene degradation and the highest absolute and relative *bssA* abundances of all the sites and conditions studied (Figure 4-2). During the first four days of incubation, in either the presence or absence of ethanol, numbers of *bssA* copies increased over 100-fold (from ca. 10^6 copies to between 10^8 and 10^9 copies; Figure 4-2). Such an increase in the population of denitrifying, toluene-degrading bacteria (on the order of 10^8 cells) is plausible, given the reported range of cell yields for denitrifying strains (ca. 50–100 g cells/mol toluene; Spormann and Widdell, 2000), the mass of toluene consumed in these microcosms (Figure 4-2[A]), and the approximate mass of a bacterium (Neidhardt *et al.*, 1996). In addition, there was some xylene consumption during the first four days (Chapter 3, Alvarez *et al*., 2001), which could have further supported the growth of bacteria containing the *bssA* gene. The increase in *bssA* copies represents a maximum possible increase in the numbers of bacteria carrying this gene, since individual bacteria could have more than one copy of *bssA* per cell, particularly if they were in an exponential growth phase. If there were on average one copy of *bssA* per cell, the observed hundred- to thousand-fold increase in four days is plausible; reported doubling times of denitrifying bacteria growing on toluene (Spormann and Widdel, 2000) suggest that a thousand-fold increase (i.e., ten doublings) would occur in $\leq$2.5 days. Thus,

the increases in *bssA* copies based on the RTQ-PCR method are plausible and help to explain the high toluene degradation rate observed.

In contrast to results for the Travis AFB, denitrifying microcosms, there were only relatively small changes (two- to four-fold) in *bssA* abundances in microcosms incubated under all other conditions (Figures 4-3 to 4-9). Although such differences may be within the range of experimental error, they are nonetheless informative. These results indicate that, even after degradation of enough toluene to potentially support the growth of ca. 10^7 or 10^8 cells, no such increases in the abundances of *bssA* copies were observed. One possible explanation for these results is that the toluene-degrading bacteria were in a physiological state in which they metabolized toluene but didn't use it for growth. Since the artificial groundwater used in these microcosms wasn't amended with vitamins, growth may have been nutrient-limited in certain cases. Another possible explanation is that the primers and probe used for *bssA* detection did not efficiently match the actual *bssA* sequences of the toluene-degrading bacteria in particular microcosms. Inefficiency of the *bssA* primers and probe is not very likely for denitrifying conditions, as the primers and probe were based on a consensus region of *bssA* sequences from four different denitrifying strains; however, the primers and probe were very inefficient at detecting *bssA* from a sulfate-reducing, toluene-degrading bacterium. The design of more universally efficient *bssA* primers and probes must await the availability of sequence information from a wider range of anaerobic, toluene-degrading bacteria.

Ethanol had no clear effect on the abundance of *bssA* copies in any of the microcosms analyzed. However, in most cases, a change in numbers of *bssA* copies would not be expected because ethanol had no discernible effect on toluene degradation kinetics (Figures 4-2[A] and 4-9[A]), or if it did have an effect, it was probably related to the depletion of electron acceptors rather than to bacterial population shifts (Figures 4-4[A], 4-5[A], 4-7[A], and associated text). The one very clear case of an effect of ethanol on toluene degradation was in Northwest Terminal microcosms incubated under denitrifying conditions (Figure 4-6[A]); ethanol promoted toluene degradation in these microcosms. The most likely explanation is that ethanol degradation fortuitously increased the populations of toluene-degrading bacteria. The fact that numbers of *bssA* copies were not clearly higher after ethanol degradation suggests that, if such a population increase occurred, it was within the range of experimental error (i.e., within a factor of two to four) and was not of the scale observed in Travis AFB microcosms under denitrifying conditions (Figure 4-2[B]). This interpretation indicates that ethanol could promote relatively minor changes in microbial ecology that could result in major changes in hydrocarbon degradation. Conversely, analyses of 16S rDNA copies indicated that ethanol could have marked effects on microbial ecology (e.g., 5- to 10-fold increases in total eubacterial populations) that did not result in discernible changes in hydrocarbon degradation (e.g., Figures 4-2, 4-3, and 4-9). In one case (Sacramento microcosms incubated under denitrifying conditions; Figure 4-8), ethanol resulted in both an increase in 16S rDNA copies and an enhancement in toluene degradation rate.

When considering the effects of ethanol in this study, it is very important to note that the study design intentionally precluded a major potential effect of ethanol on the microbial ecology of hydrocarbon-degrading bacteria, namely, the successive depletion of electron acceptors and establishment of electron-accepting conditions characterized by slower toluene-degrading species (e.g., methanogenic conditions). This effect was addressed by column studies reported in Chapter 3 (Alvarez *et al*., 2001) and by previous microcosm studies (e.g., Corseuil *et al.*, 1998).

Although not directly addressed, the depletion of electron acceptors during ethanol degradation and its effect on toluene-degrading bacteria could be extrapolated from the results of this study. For example, nitrate depletion coupled with ethanol degradation at the Travis AFB site would be expected to eliminate the electron-accepting conditions conducive to high growth yields and rapid toluene degradation rates (i.e., denitrifying conditions).

Analyses based on the *bssA* gene are an estimate of the population of anaerobic, toluene-degrading bacteria (depending on how many copies of *bssA* actually occur in each cell) and of the *potential* for anaerobic toluene degradation. Some of the results in this study are a reminder that genetic potential is not always realized. To illustrate, Sacramento aquifer material (before incubation) had the highest absolute and relative numbers of *bssA* copies of any aquifer material analyzed (compare Controls in Figures 4-2[B], 4-8[B], and 4-9[B]). However, the toluene degradation rates of Sacramento denitrifying microcosms were comparable to those of Tracy denitrifying microcosms, which had by far the lowest absolute numbers of *bssA* copies. The method presented in this study could be modified to quantify the expression of *bssA* by extracting mRNA and using reverse transcription PCR. Although an mRNA-based approach would offer the advantage of quantifying expression, it would yield a less certain assessment of population than the DNA approach used in this study (because there would be no way to estimate numbers of mRNA copies per cell), and would entail the logistical problems associated with the manipulation of mRNA (which is less stable than DNA). Ideally, both DNA and mRNA approaches could be used to assess *bssA*-carrying populations as well as *bssA*-expressing populations. In any case, the results of this study suggest that the RTQ-PCR method for *bssA* could provide insights into the *in situ* microbial ecology of fuel-contaminated aquifers.

4.9. Conclusions

The following conclusions are based on the findings of this study:

- The RTQ-PCR method developed for this study was successful at determining population trends that were consistent with observed degradation activity. The most convincing results were for Travis AFB microcosms incubated under denitrifying conditions; these microcosms clearly had the most rapid toluene degradation and the highest *bss*A abundances (representative of anaerobic toluene-degrading bacterial populations) of all the sites and conditions studied.
- Ethanol had no clear effect on the abundance of *bssA* copies (representative of anaerobic toluene-degrading bacterial populations) in any of the microcosms analyzed. However, under the conditions tested (i.e., with electron acceptors supplied in excess), a change in numbers of *bssA* copies would not be expected because ethanol often had no discernible effect on toluene degradation activity.
- The one very clear case of an effect of ethanol on toluene degradation was in Northwest Terminal microcosms incubated under denitrifying conditions; ethanol promoted toluene degradation in these microcosms. In the presence of ethanol, toluene was mostly degraded within 15 days, whereas in the absence of ethanol, there was no apparent degradation in over 50 days. The most likely explanation is that ethanol degradation fortuitously increased the populations of toluene-degrading bacteria. The fact that numbers of *bssA* copies were not higher after ethanol degradation suggests that, if such a

population increase occurred, it was within the range of experimental error. This interpretation indicates that ethanol could promote relatively minor changes in microbial ecology that could result in major changes in hydrocarbon degradation.

- Ethanol was occasionally found to decrease the relative populations of anaerobic hydrocarbon-degrading bacteria. In some cases, ethanol was associated with marked (e.g., nine-fold) increases in total eubacterial populations but no discernible changes in *bssA* abundances or in hydrocarbon degradation activity. This indicates that ethanol, which is generally more degradable than BTEX compounds under anaerobic conditions, can disproportionately support the growth of bacteria that aren't anaerobic hydrocarbon degraders.
- Of the anaerobic electron-accepting conditions tested, denitrifying conditions were clearly the most supportive of anaerobic hydrocarbon degradation. Travis AFB microcosms incubated under denitrifying conditions had the most rapid toluene degradation and the highest *bssA* abundances of all the sites and conditions studied. Over the first four days of incubation, during which time most of the toluene and ethanol (when present) had been consumed, numbers of *bssA* copies increased 100- to 1000-fold (from ca. 10^6 copies to between 10^8 and 10^9 copies per 50-mL microcosm). For Travis AFB microcosms incubated under sulfate-reducing, ferric iron-reducing, and methanogenic conditions, toluene degradation was slower and no comparable increases in *bssA* copies were detected.
- Analyses based on the *bssA* gene are an estimate of the population of anaerobic, toluene-degrading bacteria as well as the *potential* for anaerobic toluene degradation. Some of the results in this study are a reminder that genetic potential is not always realized. To illustrate, Sacramento aquifer material (before incubation) had the highest absolute numbers of *bssA* copies of any aquifer material analyzed (10^6 to 10^7 copies per 50-mL microcosm). However, the toluene degradation rates of Sacramento denitrifying microcosms were comparable to those of Tracy denitrifying microcosms, which had by far the lowest absolute numbers of *bssA* copies (10^4 to 10^5 copies per 50-mL microcosm).
- To simplify data interpretation, this study did not address all possible effects of ethanol on intrinsic BTEX degradation. The study was intentionally designed to preclude a major potential effect of ethanol, namely, the successive depletion of electron acceptors and establishment of electron-accepting conditions characterized by slower hydrocarbon-degrading species (e.g., methanogenic conditions). However, this effect could be extrapolated from the results. For example, nitrate depletion coupled with ethanol degradation at the Travis AFB site would be expected to eliminate the electron-accepting conditions conducive to high growth yields and rapid toluene degradation rates (i.e., denitrifying conditions).

4.10. Recommendations

The following recommendations are based on the findings of this study:

- To evaluate whether these laboratory microcosm results can be extrapolated to actual gasohol releases at LUFT sites, this molecular ecology approach should be used to

characterize the spatial and temporal changes in subsurface microbial communities as a result of controlled gasohol release field experiments.

- The RTQ-PCR method for *bssA* should be optimized for physiological groups in addition to denitrifying bacteria. Although primers and a probe were developed for toluene-degrading, sulfate-reducing bacteria as well as denitrifying bacteria in this study, logistical constraints prevented optimization of the sulfate-reducing system.
- The scope of the RTQ-PCR method should be expanded from gene presence to gene expression (i.e., using mRNA and reverse transcription PCR). This would allow for the study of how ethanol may affect hydrocarbon-degrading activity, which would complement data from the existing method on how ethanol may affect populations of hydrocarbon-degrading bacteria.
- If enhanced *in situ* bioremediation is considered at sites contaminated with gasohol, addition of nitrate as an electron acceptor could produce positive results. Denitrifying conditions were most favorable for anaerobic BTEX degradation for all sites tested, and, for Travis AFB microcosms, supported the rapid growth of toluene-degrading bacteria. These results are consistent with the findings of other BTEX biodegradation studies. Nitrate addition is easier to implement than oxygen addition because nitrate is much more soluble in water and is not a gas. Concerns regarding the use of nitrate include the following: (a) it is regulated in groundwater, (b) nitrogen gas formed by denitrification could create bubbles and disrupt groundwater flow, and (c) the degradation of benzene, the most toxic of the BTEX compounds, may not occur under denitrifying conditions at all sites. These concerns need not disqualify *in situ* nitrate addition, as added nitrate concentrations could be kept below regulatory guidelines and *in situ* benzene degradation cannot be guaranteed under any electron-accepting conditions.

4.11. References

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Figures

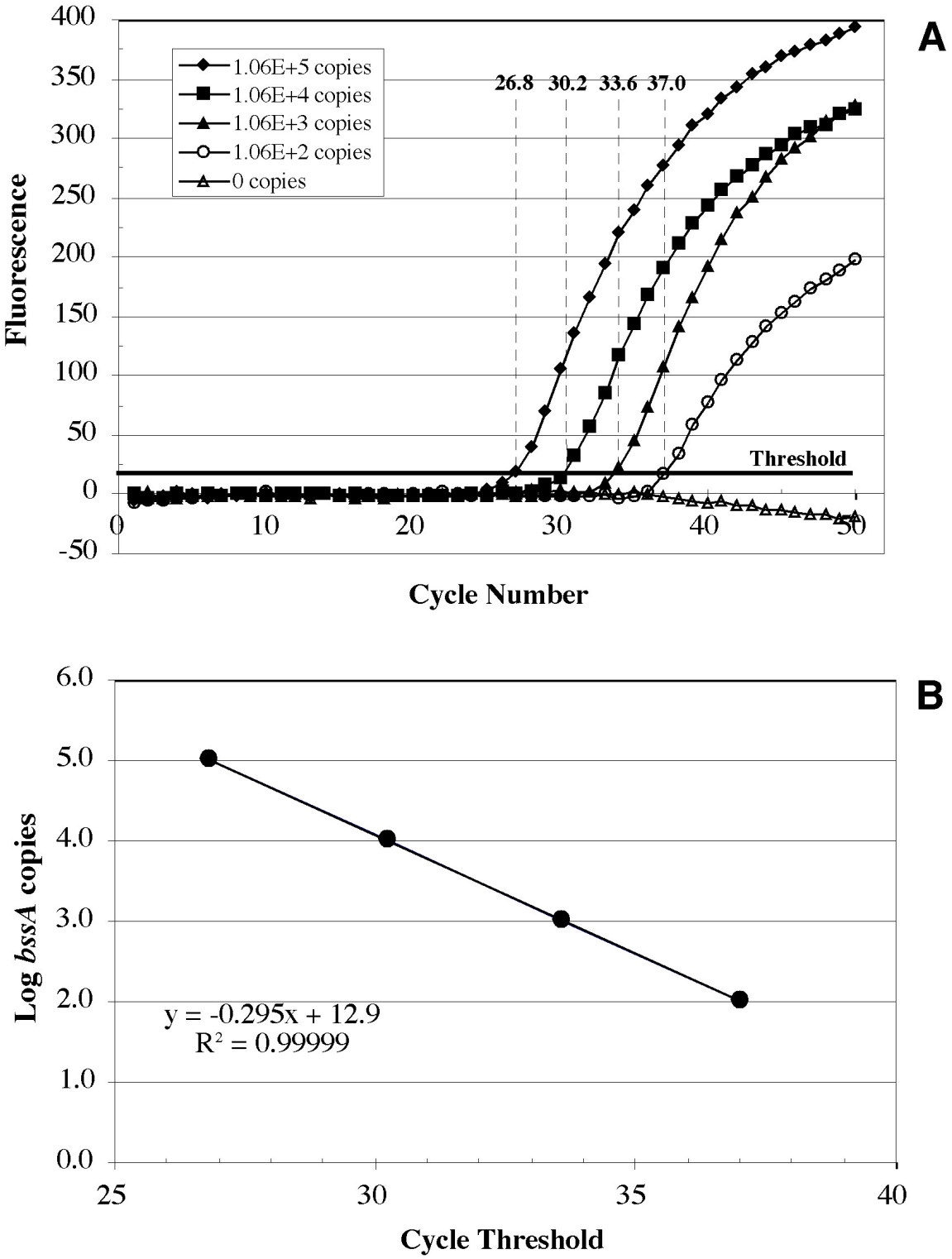

Figure 4-1. **Example of calibration data for *bssA* (using DNA from *Thauera aromatica* strain K172).** (A) Fluorescence versus PCR cycle number for four *bssA* standard solutions and a no-template control. The threshold level (10 times the standard deviation of the background noise) is denoted by a line and the cycle threshold (C_t) values are marked for each curve (above vertical dashed lines). (B) Log_{10} of *bssA* copies versus cycle threshold for the data presented in (A).

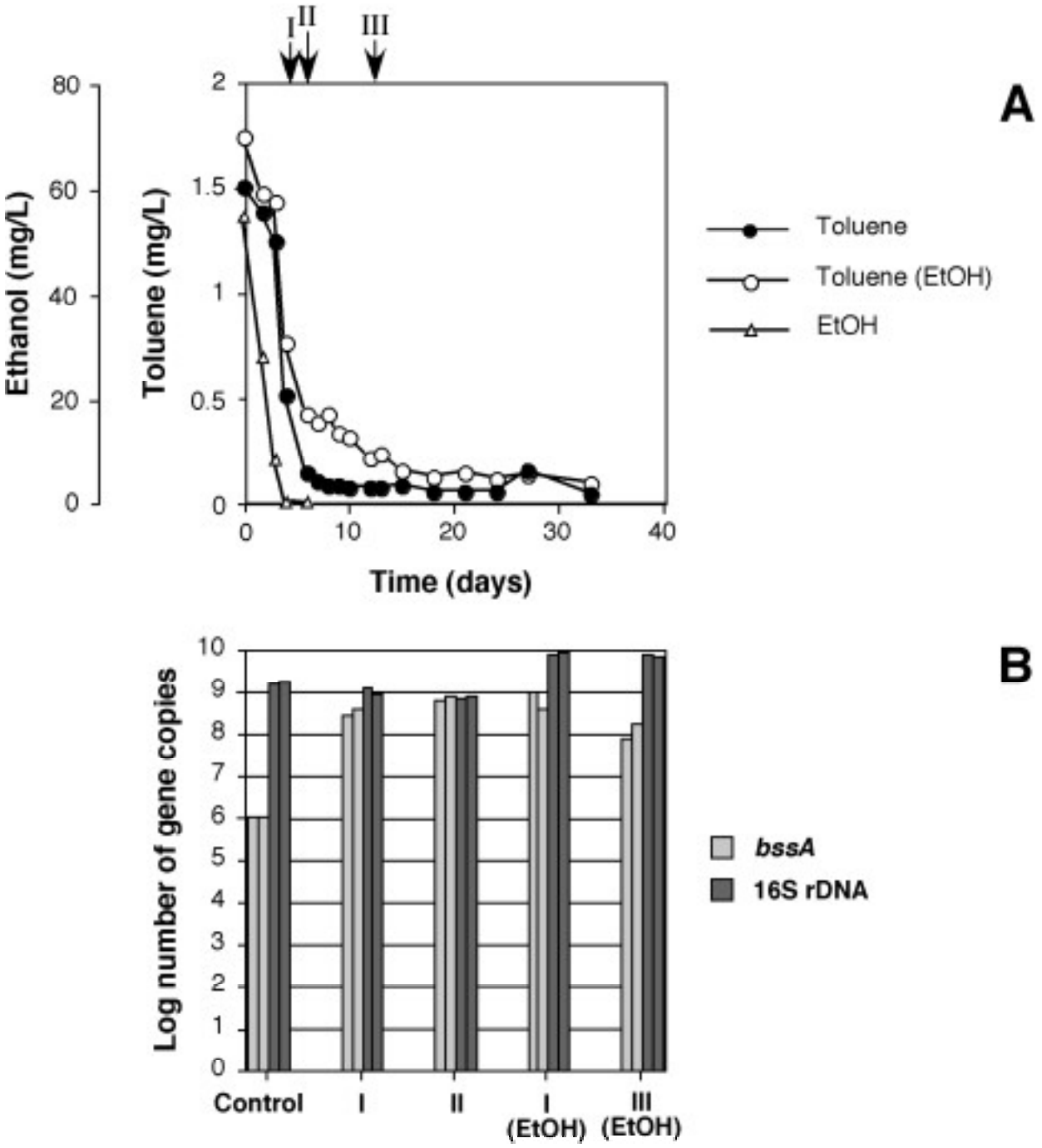

Figure 4-2. **Results for microcosms inoculated with aquifer sediment from Travis AFB and incubated under denitrifying conditions.** (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. I, II, and III designate sampling times that were based on ethanol or toluene degradation (see Materials and Methods). (B) Numbers of copies (per 50-mL microcosm, expressed as a base 10 logarithm) of *bssA* (a gene associated with anaerobic toluene degradation) or 16S rDNA, based on RTQ-PCR analysis. All individual replicates are shown. "Control" represents samples taken on Day 0; I, II, and III correspond to samples taken at the times shown in (A).

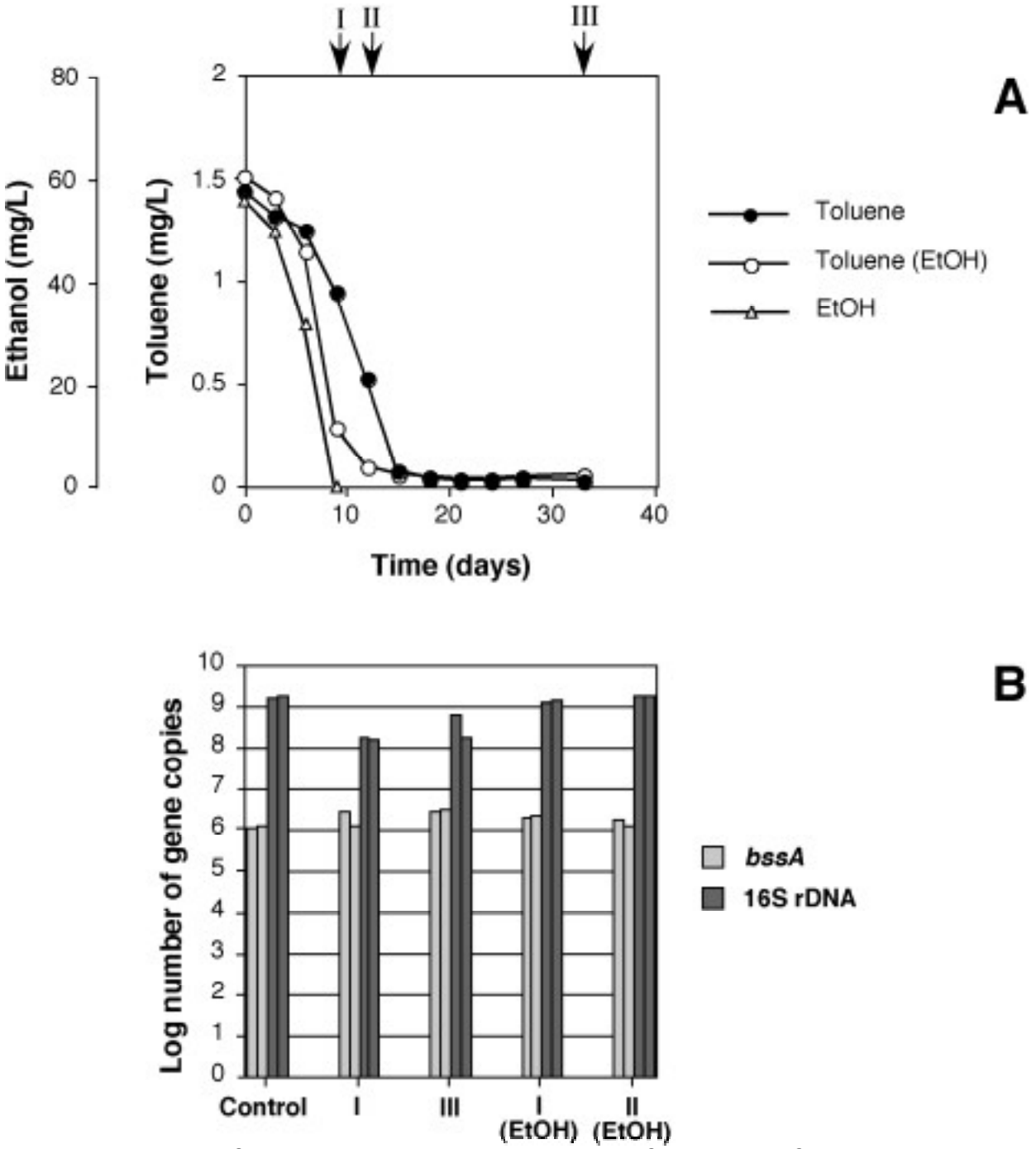

Figure 4-3. Results for microcosms inoculated with aquifer sediment from Travis AFB and incubated under sulfate-reducing conditions. (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. I, II, and III designate sampling times that were based on ethanol, toluene, or xylene degradation (see Materials and Methods). (B) Numbers of copies (per 50-mL microcosm, expressed as a base 10 logarithm) of *bssA* (a gene associated with anaerobic toluene degradation) or 16S rDNA, based on RTQ-PCR analysis. All individual replicates are shown. "Control" represents samples taken on Day 0; I, II, and III correspond to samples taken at the times shown in (A).

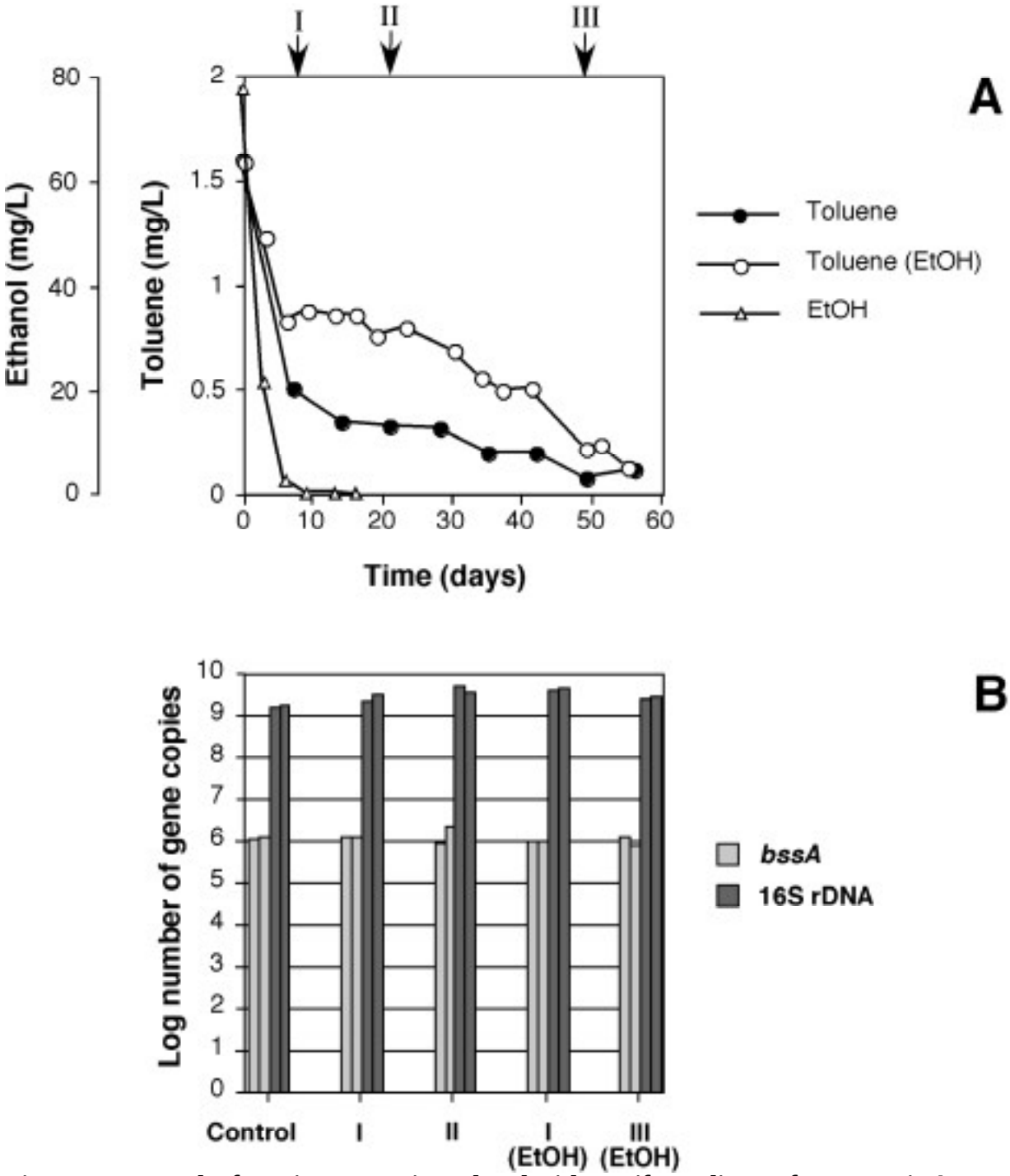

Figure 4-4. Results for microcosms inoculated with aquifer sediment from Travis AFB and incubated under putative methanogenic conditions. (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. I, II, and III designate sampling times that were based on ethanol or toluene degradation (see Materials and Methods). (B) Numbers of copies (per 50-mL microcosm, expressed as a base 10 logarithm) of *bssA* (a gene associated with anaerobic toluene degradation) or 16S rDNA, based on RTQ-PCR analysis. All individual replicates are shown. "Control" represents samples taken on Day 0; I, II, and III correspond to samples taken at the times shown in (A).

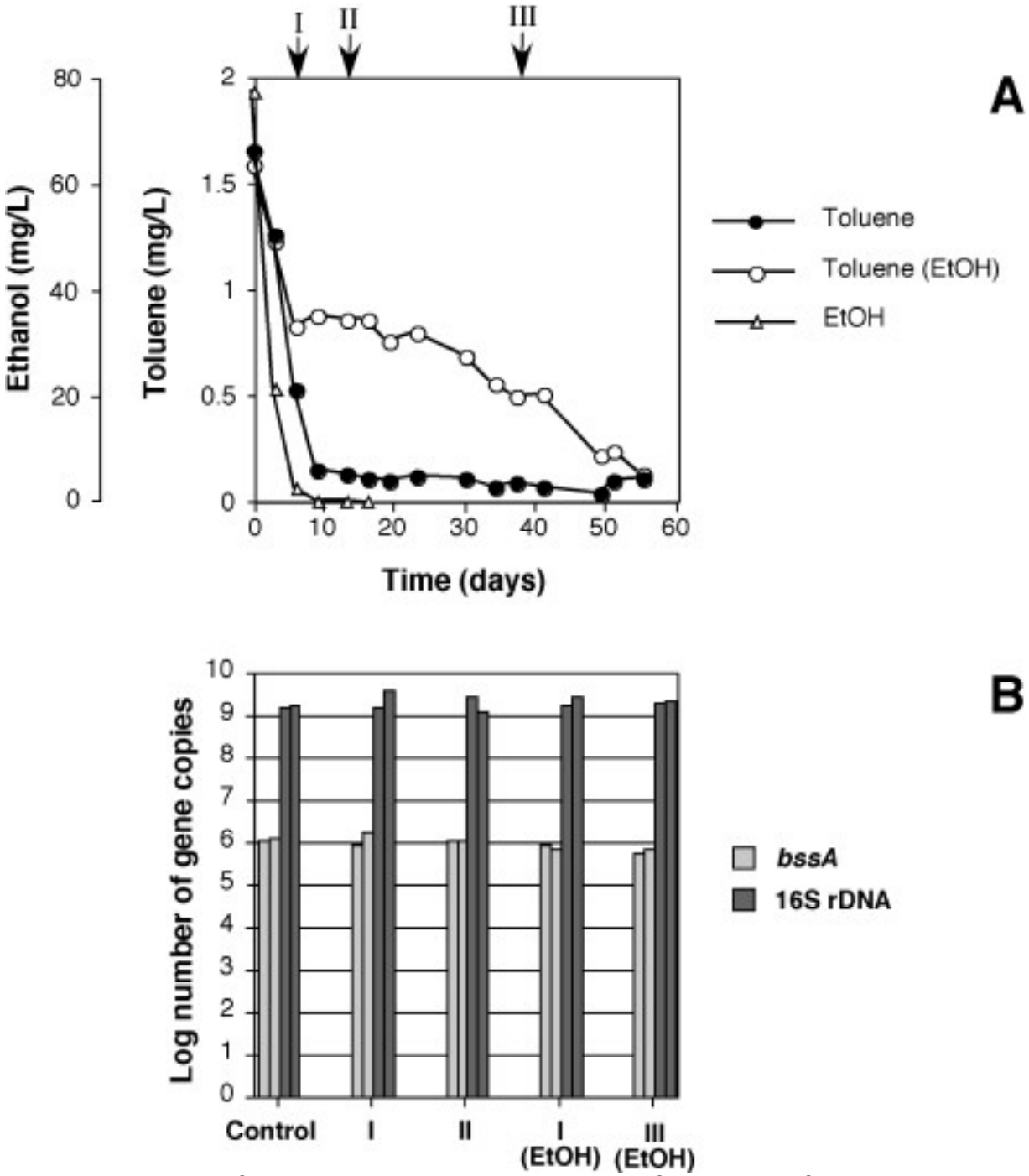

Figure 4-5. Results for microcosms inoculated with aquifer sediment from Travis AFB and incubated under ferric iron-amended conditions. (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. I, II, and III designate sampling times that were based on ethanol or toluene degradation (see Materials and Methods). (B) Numbers of copies (per 50-mL microcosm, expressed as a base 10 logarithm) of *bssA* (a gene associated with anaerobic toluene degradation) or 16S rDNA, based on RTQ-PCR analysis. All individual replicates are shown. "Control" represents samples taken on Day 0; I, II, and III correspond to samples taken at the times shown in (A).

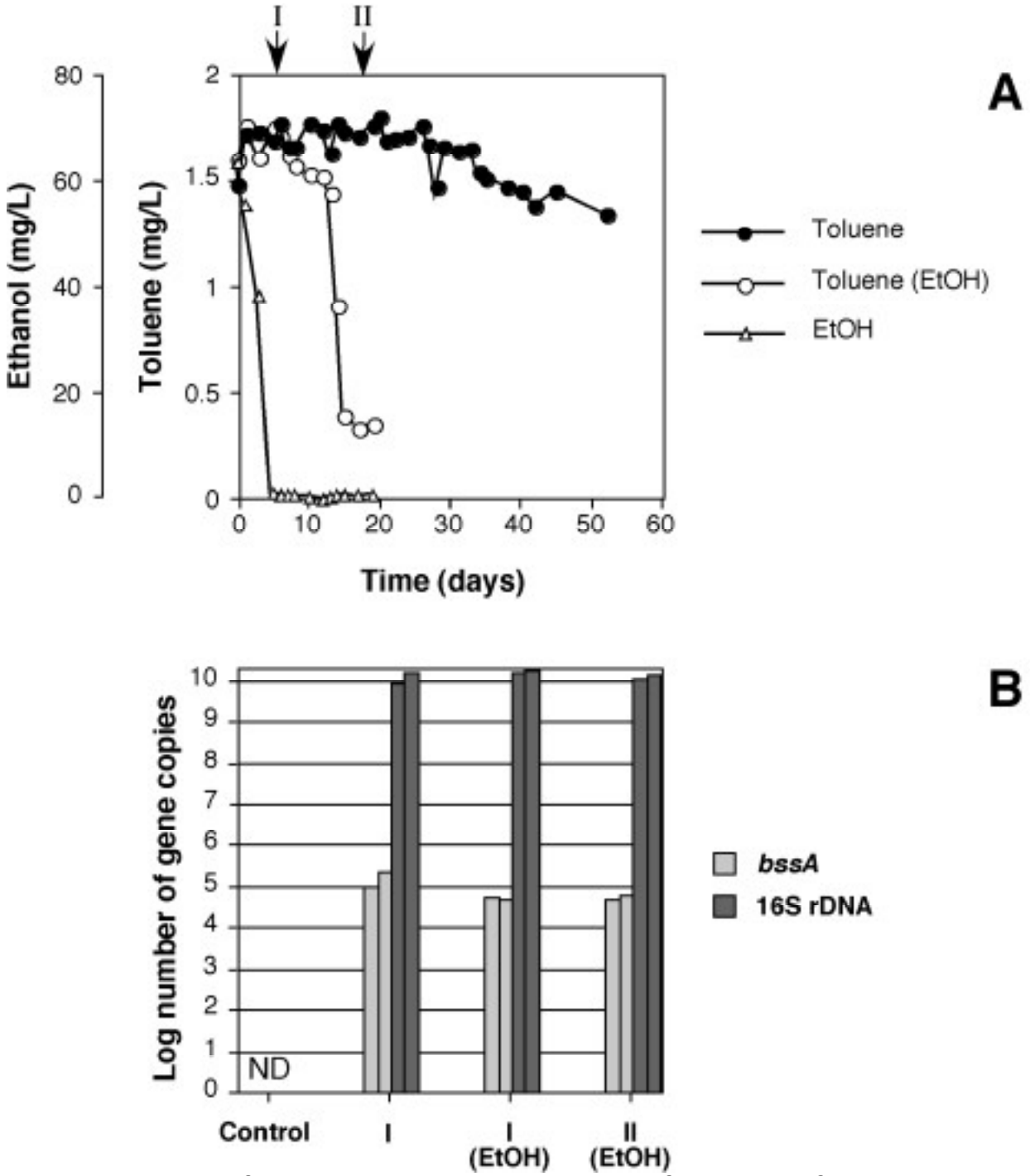

Figure 4-6. Results for microcosms inoculated with aquifer sediment from the Northwest Terminal and incubated under denitrifying conditions. (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. I and II designate sampling times that were based on ethanol or toluene degradation (see Materials and Methods). (B) Numbers of copies (per 50-mL microcosm, expressed as a base 10 logarithm) of *bssA* (a gene associated with anaerobic toluene degradation) or 16S rDNA, based on RTQ-PCR analysis. All individual replicates are shown. "Control" represents samples taken on Day 0 (ND, not detected); I and II correspond to samples taken at the times shown in (A).

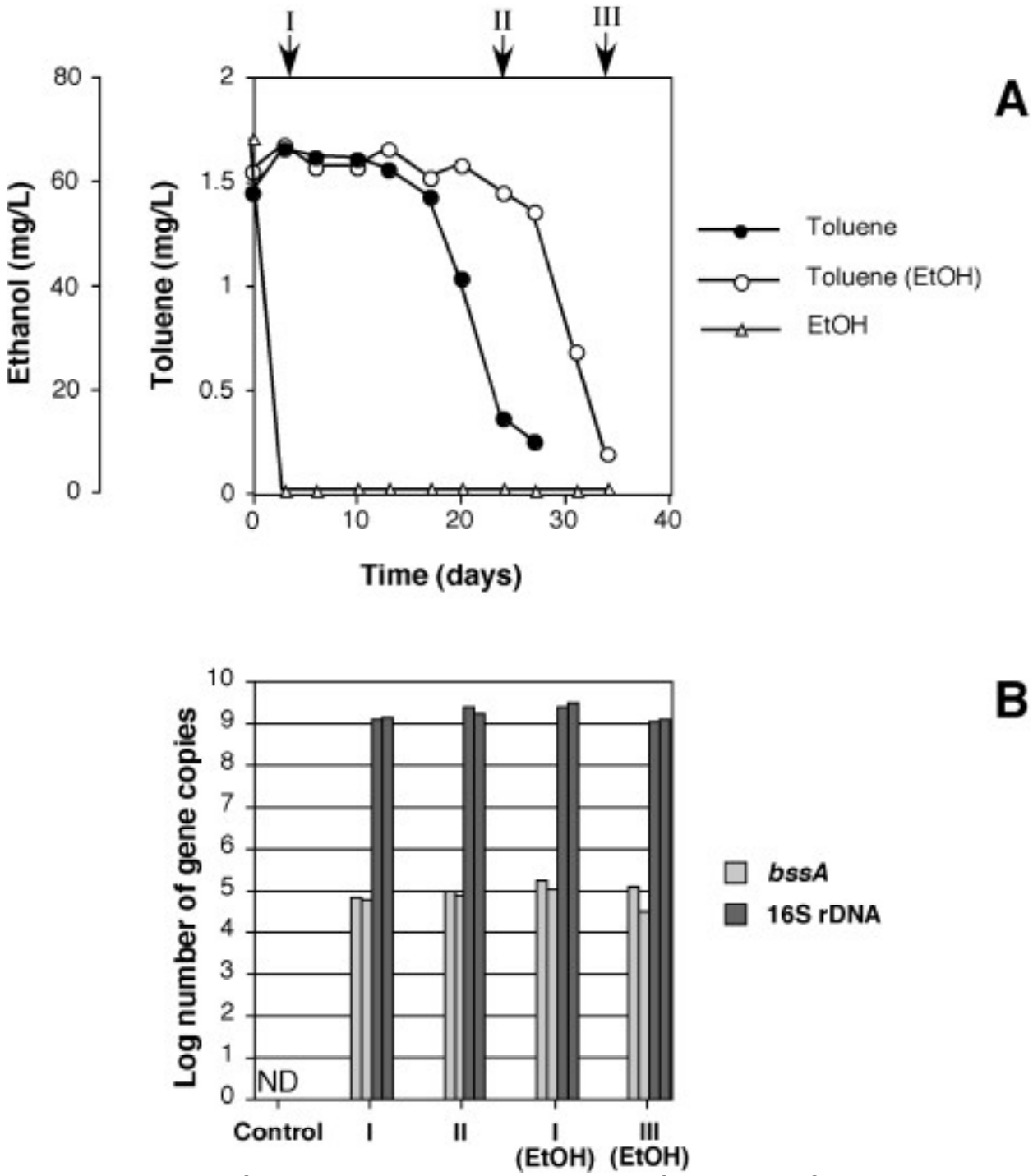

Figure 4-7. Results for microcosms inoculated with aquifer sediment from the Northwest Terminal and incubated under sulfate-reducing conditions. (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. I, II, and III designate sampling times that were based on ethanol or toluene degradation (see Materials and Methods). (B) Numbers of copies (per 50-mL microcosm, expressed as a base 10 logarithm) of *bssA* (a gene associated with anaerobic toluene degradation) or 16S rDNA, based on RTQ-PCR analysis. All individual replicates are shown. "Control" represents samples taken on Day 0 (ND, not detected); I, II, and III correspond to samples taken at the times shown in (A).

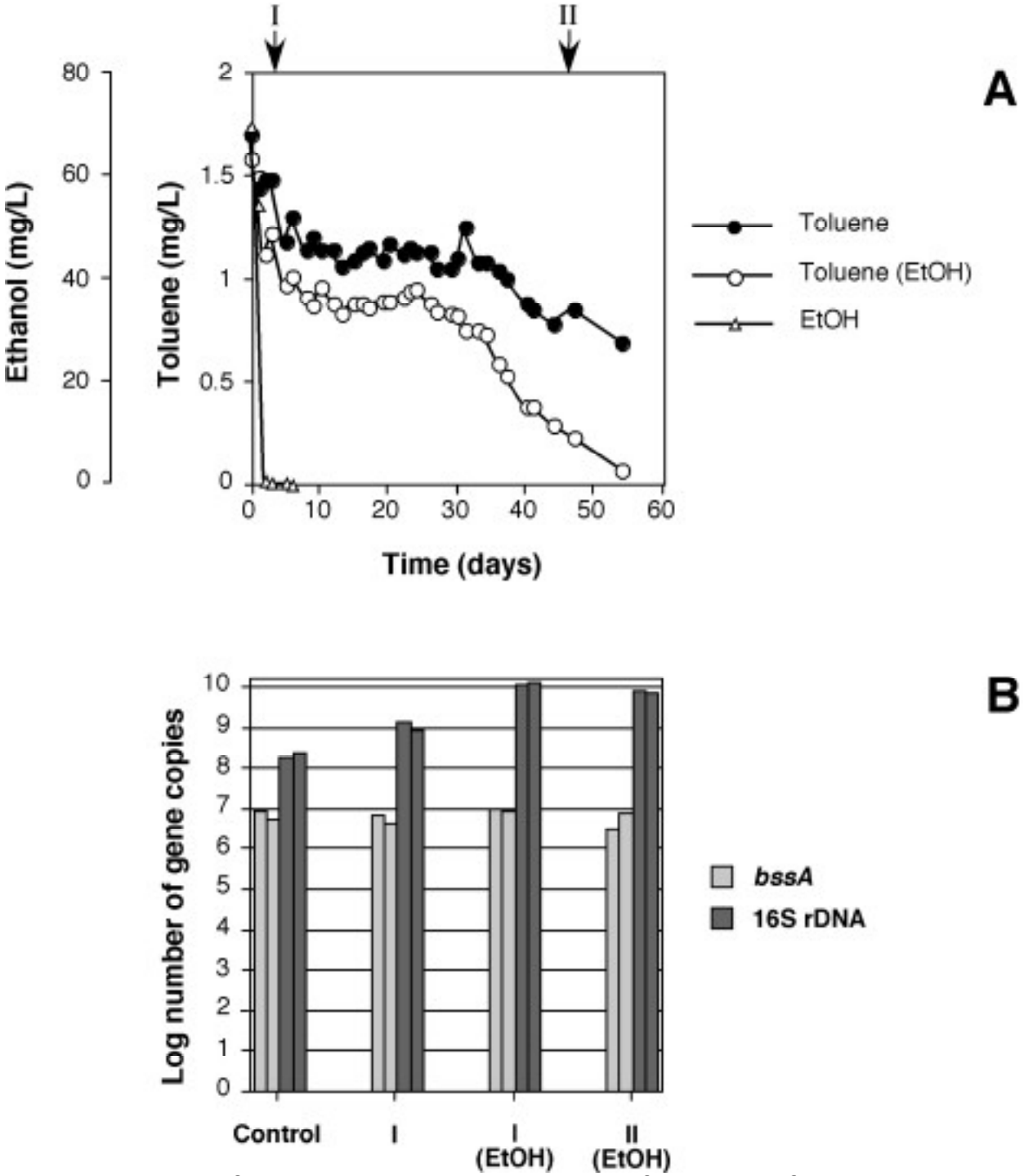

Figure 4-8. Results for microcosms inoculated with aquifer sediment from the Sacramento site and incubated under denitrifying conditions. (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. I and II designate sampling times that were based on ethanol or toluene degradation (see Materials and Methods). (B) Numbers of copies (per 50-mL microcosm, expressed as a base 10 logarithm) of *bssA* (a gene associated with anaerobic toluene degradation) or 16S rDNA, based on RTQ-PCR analysis. All individual replicates are shown. "Control" represents samples taken on Day 0; I and II correspond to samples taken at the times shown in (A).

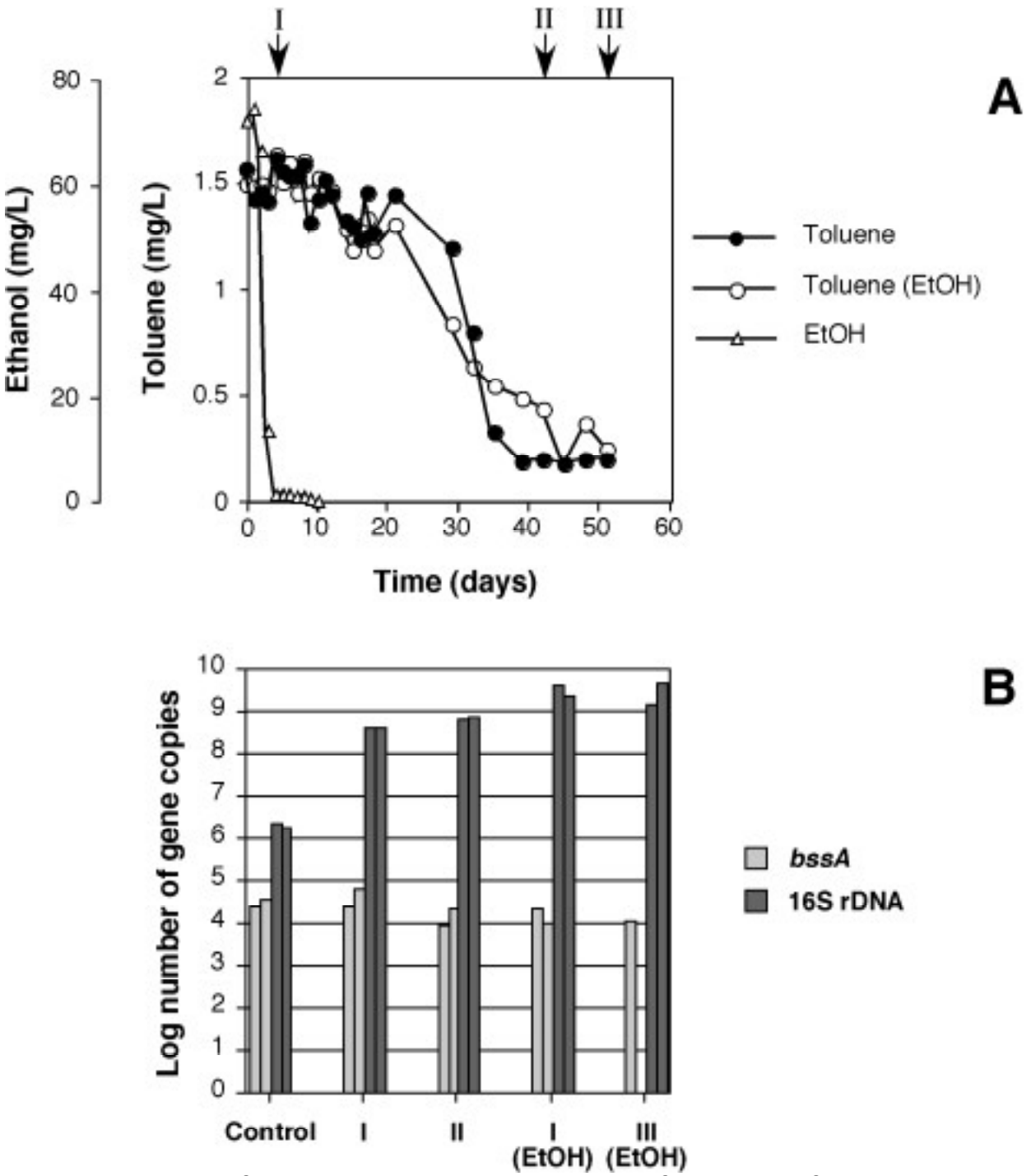

Figure 4-9. Results for microcosms inoculated with aquifer sediment from the Tracy site and incubated under denitrifying conditions. (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. I, II, and III designate sampling times that were based on ethanol or toluene degradation (see Materials and Methods). (B) Numbers of copies (per 50-mL microcosm, expressed as a base 10 logarithm) of *bssA* (a gene associated with anaerobic toluene degradation) or 16S rDNA, based on RTQ-PCR analysis. All individual replicates are shown. "Control" represents samples taken on Day 0; I, II, and III correspond to samples taken at the times shown in (A).